Location of Binding Sites on Common Type 1 Fimbriae from *Escherichia coli*

By GRACE SWEENEY* AND JOHN H. FREER

Department of Microbiology, University of Glasgow, Alexander Stone Building,
Garscube Estate, Bearsden, Glasgow G61 1QH

(Received 4 October 1978)

Common type 1 fimbriae were isolated from *Escherichia coli* and their length distribution profile was determined before and after treatment with ultrasound. As fimbriae were shortened, so their haemagglutinating capacity decreased, but their ability to bind to erythrocytes did not decrease to the same extent. Isolated fimbriae did not agglutinate inside-out vesicles prepared from horse erythrocytes or liposomes, suggesting that the binding mechanism was not based on non-specific hydrophobic interactions. The results support a lateral rather than a terminal location for the fimbrial binding site responsible for haemagglutination.

INTRODUCTION

A renewed interest in bacterial surface structures classified as fimbriae (Duguid *et al.*, 1955) or pili (Brinton, 1959) has stemmed from the realization that they may control, or at least strongly influence, the initial phase of infection in some diseases caused by Gram-negative bacteria. In many instances colonization is thought to be preceded by an absorption phase involving specific binding of bacterial surface structures to receptor sites on the cell membranes of host tissues (Jones, 1977).

Common type 1 fimbriae occur in the Enterobacteriaceae and have been widely reported in the genera *Escherichia*, *Salmonella* and *Shigella* (Duguid & Gillies, 1957; Hashimoto *et al.*, 1963*a, b*). The presence of common type 1 fimbriae on bacterial cells confers on them the ability to agglutinate erythrocytes of guinea pig or horse, a process which is reversed by low concentrations of α -D-mannopyranose or α -D-methyl mannopyranoside. This phenomenon is termed mannose-sensitive haemagglutination. In contrast, the presence of colonization factor antigen, a pilus-like structure present on certain human enterotoxigenic strains of *Escherichia coli* causing infant diarrhoea (Evans *et al.*, 1975), confers on these strains a mannose-resistant haemagglutinating capacity towards human erythrocytes (Evans *et al.*, 1977).

The increase in adhesiveness of bacteria carrying common type 1 fimbriae can be demonstrated by their increased affinity towards a variety of mammalian cells, plant cells and fungal cells (Duguid & Gillies, 1957; Duguid *et al.*, 1966; Hashimoto *et al.*, 1963*a, b*), to yeast mannan (Old, 1972) and to 'polystyrene latex' spheres (Brinton, 1965). In all these cases, with the possible exception of the last, adhesion appears to involve specific binding between sites on the fimbriae and receptors which are thought to include saccharide moieties on the cell surface.

The structure of common type 1 fimbriae has been investigated in detail by Brinton (1965) who reported that the filaments are composed of a single subunit species arranged in a low pitch helix to form a hollow filament of approximately 7 nm outside diameter, with a hollow core of 2 to 2.5 nm diameter.

* Present address: Department of Bacteriology, The Royal Infirmary, Glasgow.

While there appears little doubt that common type 1 fimbriae are the organelles of attachment involved in mannose-sensitive adhesion of bacteria to erythrocytes and a variety of other substrates, no information is available which defines the location of the binding site on the fimbriae. From the results of Brinton (1965) using 'polystyrene latex' spheres it appears that the binding site is terminal, since fimbriae were observed attached to latex spheres only by their tips. However, more recently Salit & Gotschlich (1977) reported that type 1 fimbriate cells of *E. coli* attached to the surface of monkey kidney cells via fimbriae which were in contact with the kidney cell surface over considerable lengths.

This paper presents data which are consistent with laterally located binding sites in common type 1 fimbriae.

METHODS

Organism and growth conditions. *Escherichia coli* (NCTC 8623) serotype O125:K70(B15):H19 was obtained from the National Collection of Type Cultures, Colindale, London.

Preparation of fimbriae. Fimbriae were prepared from cells grown in static culture for 48 h at 37 °C in 1 litre Fernbach flasks, each containing 350 ml nutrient broth no. 2 (Oxoid). The method used was based on that of Novotny *et al.* (1969). Formalin-treated cultures were centrifuged at 5000 *g* for 15 min and the sedimented cells were washed once in phosphate buffered saline (PBS; 5 mM-NaH₂PO₄/Na₂HPO₄ buffer, pH 7.3, containing 0.145 M-NaCl). The pellet was resuspended in PBS to a cell concentration of 4×10^8 ml⁻¹ and blended at 4 °C in an M.S.E. homogenizer for 2 min at 9500 rev. min⁻¹. This procedure detached most of the flagella filaments and sex pili, as assessed by electron microscopy. The blended cell suspension was centrifuged at 10000 *g* for 15 min, the supernatant was discarded and the pellet was resuspended in PBS and blended at 14000 rev. min⁻¹ for 2 min at 4 °C. Most of the cells were subsequently removed from the blended suspension by centrifugation at 10000 *g* for 15 min. The supernatant fluid containing fimbriae was subjected to two cycles of centrifugation at 30000 *g* for 30 min, which sedimented remaining whole cells, lysed cells and cell debris. The supernatant fluid was then centrifuged at 150000 *g* for 120 min; the fimbriae formed a transparent gelatinous pellet which was resuspended in PBS or distilled water to a concentration of about 1 mg protein ml⁻¹. Sodium azide (60 µM) was added as an inhibitor of bacterial growth and the preparation was stored at 4 °C.

Treatment with ultrasound. Suspensions of fimbriae were subjected to ultrasound using an M.S.E. ultrasonic disintegrator with an exponential probe. Suspensions on ice were exposed for periods of 30 s with alternating cooling periods of 30 s, up to a total exposure period of 12 min.

¹²⁵I-Labeling of fimbriae. Fimbriae were labelled with ¹²⁵I using the procedure of Gow & Wardlaw (1975) which was based on that described by Hubbard & Cohn (1972). The reaction mixture consisted of 10 milliunits lactoperoxidase (Sigma), 10 milliunits glucose oxidase (Sigma), 10 µCi carrier-free Na¹²⁵I (The Radiochemical Centre, Amersham), 5 µM-glucose and 1 to 2 mg fimbrial protein. Following incubation of the complete reaction mixture for 1 h at 37 °C, the preparation was dialysed against three 5 l volumes of distilled water containing 10 µM-sodium thiosulphate during 24 h. After dialysis the labelled fimbriae were stored at 4 °C with 50 µM-NaN₃ as a growth inhibitor. The radioactivity of 100 µl samples of suspension was measured in a Nuclear Enterprises 8312 automatic β-γ spectrometer. Using this labelling technique the specific activity was approx. 5×10^5 c.p.m. (mg protein)⁻¹. Labelled fimbriae retained their mannose sensitivity and erythrocyte specificity in haemagglutination tests.

Haemagglutination (HA) tests. These were done using the Cooke Engineering microtitre system (Flow Laboratories, Irvine, Scotland). Doubling dilutions of fimbriae were prepared in 0.05 ml PBS and 0.05 ml of a 1 % (v/v) suspension of washed horse erythrocytes was added to each well. After thorough mixing (5 min), the trays were refrigerated overnight and the titre was recorded as the reciprocal of the highest dilution of fimbriae which caused agglutination of the erythrocytes.

Protein estimation. Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Measurement of fimbrial length. The length of fimbriae was measured on enlarged electron micrographs taken of negatively stained preparations of fimbriae. Filament lengths were measured using a cartographer's wheel. One thousand filaments from each preparation were measured and the results were plotted as histograms to show the length distribution.

Electron microscopy. Negative staining of fimbriae was done using 2 % (w/v) potassium phosphotungstate. Equal volumes of fimbrial suspension and negative stain were mixed and applied to carbon-coated Formvar films. The excess fluid was removed with a filter paper point and the remaining film was allowed to dry. Grids were examined in a Philips EM300 electron microscope.

Preparation of membrane vesicles. Inside-out vesicles were prepared from washed horse erythrocytes by

Fig. 1. Electron micrograph of a negatively stained preparation of freshly isolated fimbriae. Bar marker represents 200 nm.

the method of Steck (1974). The small amount of right-side-out vesicles contaminating the preparation was removed by adding concanavalin A ($50 \mu\text{g ml}^{-1}$; Sigma) to the suspension and, after 5 min gentle mixing, separating the small amount of agglutinated membrane by centrifugation at $600 g$ for 10 min. This process was repeated until no further agglutination occurred. The inside-out vesicles were then washed in $0.5 \text{ mM-NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer pH 8.0 containing $50 \text{ mM-}\alpha$ -methyl mannopyranoside to remove concanavalin A. The vesicles were then washed twice in $10 \text{ vol. } 0.5 \text{ mM-NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer pH 8.0 and used in place of erythrocytes in the standard haemagglutination assay.

Lipid vesicles (liposomes) were prepared according to Freer *et al.* (1968) using egg phosphatidyl choline, dicetyl phosphate and cholesterol in molar ratios of 70:20:10. The vesicle preparation, in PBS, was used in place of erythrocytes in the standard haemagglutination assay at a concentration of approximately $17 \mu\text{mol lipid ml}^{-1}$. A positive agglutination control of the vesicle preparations was achieved by addition of CaCl_2 to the assay well to a final concentration of 10 mM .

RESULTS

Figure 1 shows the appearance of isolated fimbriae in negatively stained preparations examined by electron microscopy. The length distribution in a freshly isolated preparation of fimbriae was assessed from such micrographs by direct measurement (Fig. 2). The fragments which occurred with greatest frequency were those measuring between 100 and 200 nm (about a quarter of the fragments measured were in this category). Some 77% of the filament protein in the preparation was accounted for in filaments with lengths between 100 and 600 nm. When samples from the same filament preparation were subjected to ultrasound for 5 min, the length distribution profile changed markedly (Fig. 3). After sonication there were no filaments with lengths greater than 70 nm and over 70% of the filament protein was accounted for in filaments of between 5 and 35 nm in length. Over 70% of the filaments present were between 5 and 25 nm in length.

The effect of ultrasound on the ability of fimbriae to induce haemagglutination is shown in Fig. 4. There was an exponential loss of haemagglutinating activity, without any lag, accompanied by a progressive reduction in the length of the filaments (data not shown) as the sonication time was increased. After 5 min of treatment the haemagglutinating activity was completely lost.

Fig. 2

Fig. 3

Fig. 2. Histogram showing the length distribution profile and the percentage of the total protein in each length class for freshly isolated fimbriae. The percentage of the total sample protein present in each class was estimated as:

$$\frac{\text{Sum of lengths of all fimbriae measured in class}}{\text{Sum of lengths of all fimbriae measured in all classes}} \times 100.$$

The results are plotted (○) to show the sum of the percentages of the total protein present in each class and all smaller classes.

Fig. 3. Histogram showing the length distribution profile and the percentage of the total protein in each length class for a preparation of fimbriae subjected to ultrasound for 5 min. The protein percentages were estimated and plotted as in Fig. 2.

Fig. 4

Fig. 5

Fig. 4. Kinetics of loss of haemagglutinating activity of fimbriae during treatment with ultrasound.
Fig. 5. Relationship between fimbrial protein bound to erythrocytes (○) and haemagglutination titre (●) during treatment of fimbriae with ultrasound.

Figure 5 shows the relationship between the progressive reduction in the length of filaments and their ability to bind to erythrocytes. The amount of ^{125}I -labelled protein bound to erythrocytes fell only slightly during the initial sonication period when the HA titre fell markedly from 512 to 32 and thereafter remained almost constant during a further reduction in HA titre to 4. The increased period of sonication required to reduce the HA titre from 256 to 2 in Fig. 5 when compared with Fig. 4 simply reflects differences in sonication conditions including sample volume, the geometry of the container and the rate of energy input.

When liposomes or inside-out vesicles prepared from horse erythrocytes were used in place of horse erythrocytes in the microtitre haemagglutination assay, no agglutination was observed with 32 haemagglutinating units of isolated fimbriae.

DISCUSSION

The general acceptance that bacterial fimbriae and, in particular, common type 1 fimbriae are composed of identical subunits, which aggregate in a specific manner to form hollow cylinders, raises important questions relating to their mode of binding to substrates. The binding of both whole fimbriate *E. coli* NCTC 8623 and isolated type 1 fimbriae to erythrocytes displays mannose sensitivity and a similar species specificity. It is generally assumed that the same mechanism is involved in haemagglutination induced by either isolated fimbriae or whole fimbriate cells.

The location on the filament of the site of the haemagglutinin(s) or fimbrial adhesin(s) responsible for this type of binding could be terminal or lateral. The concept of a single specific binding region located at the distal tip of fimbriae is not tenable since this would make isolated fimbriae monovalent. It would then be difficult to explain the haemagglutinating capacity of isolated fimbriae without postulating binding via bundles of filaments. However, subunits in the terminal regions of either isolated fimbriae or cell-associated fimbriae must differ from those in lateral regions in the sense that different areas of the subunit surface will be exposed to potential receptors. This difference between the regions of the subunits exposed to the environment at the termini and laterally provides a rational basis for the suggestion that adhesion sites occur at each end of filaments. Since the subunits of fimbriae are thought to associate to form filaments via hydrophobic interactions, then relatively hydrophobic areas would be exposed at each terminus of an isolated filament. This may explain the observations of Brinton (1965), that isolated type 1 fimbriae stick to 'polystyrene latex' beads by their tips and not their sides, since 'polystyrene latex' would be expected to have a relatively non-polar surface. This type of adhesion via hydrophobic interactions would not be expected to display mannose sensitivity. Unfortunately no data have been published on whether or not the terminal binding of fimbriae to such beads is mannose-sensitive. We have found no evidence to suggest that a non-specific hydrophobic interaction is involved in binding of fimbriae to membranes. Inside-out vesicles prepared from horse erythrocytes are not agglutinated by isolated fimbriae. Also artificial membrane vesicles (liposomes) prepared from egg phosphatidyl choline, dicetyl phosphate and cholesterol are not agglutinated by isolated fimbriae.

If fimbriae have terminal mannose-specific binding sites, i.e. two such sites for each filament, generation of two new potential binding sites could occur each time a filament was broken. Thus, in the simplest case, if sonication caused all filaments to break, then the number of potential binding sites would double. During the initial period of sonication there should be rapid generation of new termini and a rise in the HA titre of the preparation may be expected. Superimposed on this effect would be a reduction of some of the shorter filaments in the preparation to a length which is below the minimum required for cross-linking of erythrocytes. Even assuming a relatively high minimal length requirement for cross-linking to occur, e.g. > 50 nm, if all filaments in the preparation shown in Fig. 2 were broken in half, the titre of the preparation would still be expected to rise by about 40 %

Fig. 6. Diagrams showing ¹²⁵I-labelled fimbriae binding terminally (a) and laterally (c) to receptors (●) on erythrocytes (large broken circles). Diagrams (b) and (d) show the consequences in terms of amount of protein bound if the fimbrial length is reduced fourfold. In the case of lateral binding it is assumed that each fimbrial sub-unit carries a binding site, whereas in terminal binding, sites are present only at each end of a filament.

(assuming that HA titre is directly related to the number of filaments > 50 nm in length). This is clearly not the case and no transient rise in titre was observed before the progressive fall during sonication of isolated filaments (see Fig. 4). A decrease in haemagglutinating activity with sonication was reported by Salit & Gotschlich (1977) in studies of isolated type 1 fimbriae from a strain of *E. coli* K12. The results shown in Fig. 4 support the concept that the HA titre is related to the number of filaments above a critical length. This, in turn, favours a lateral location for binding sites distributed along the length of the filaments.

Further evidence favouring this concept comes from the results of the experiment in which the amount of fimbrial protein bound to a fixed amount of erythrocytes was followed during treatment of the labelled fimbriae with ultrasound. In this instance, a fixed number of receptor sites on the erythrocytes were present in the reaction mixtures. If the filaments have terminal binding sites, then as they are reduced in length during increasing periods of exposure to ultrasound, one would not only expect a transient increase in the HA titre of the preparation but also a progressive decrease in the amount of protein bound, since each erythrocyte receptor would be binding a shorter filament. Clearly this was not the case. After a slight fall in the initial amount of protein bound, a plateau was reached and the level of bound (sedimentable) protein became almost constant even though the HA titre continued to fall. This result favours a lateral location for binding sites as discussed above. The constant level of protein bound during a progressive shortening of filaments can be explained by a mechanism such as that illustrated diagrammatically in Fig. 6. The constant number of sites are at all times fully saturated, but the filament length has been reduced in (b) and (d) to a quarter of that in (a) and (c). During treatment with ultrasound a slight loss in the amount of bound protein followed by a constant amount bound (as seen in Fig. 5) is consistent with the mechanism shown in Fig. 6 (c, d), but not with that shown in Fig. 6(a, b). In the first mechanism (Fig. 6a, b) one would expect a decrease in the amount of bound protein to one-quarter of the original value whereas in the second mechanism only a relatively small decrease in bound protein would result.

Old & Payne (1971) proposed that the fimbrial binding site (haemagglutinin) was situated at the tip of filaments attached to whole cells of *Salmonella* spp. This suggestion was based on the finding that the so-called haemagglutinin was not a major fimbrial antigen and therefore that the fimbrial tip was a likely location. In contrast, Duguid & Gillies (1958) favoured a lateral location for the fimbrial haemagglutinin. Again their conclusion was based upon immunological data. Fimbrial antibodies caused an inhibition of haemagglutination and the antibody molecules were found to be located laterally on fimbrial

filaments, rather than terminally, when cells were examined by electron microscopy. More recently Salit & Gotschlich (1977) examined the adherence of fimbriate *E. coli* K12 to monolayer cultures of monkey kidney cells and after electron microscopy of the adherent cells concluded that fimbriae adhere along their length rather than at their tips as was the case for gonococcal pili (Swanson, 1973).

Our data suggest that adherence to erythrocyte receptors occurs along the length of fimbriae. The adherence of isolated fimbriae via their tips to 'polystyrene latex' spheres as reported by Brinton (1965) and a similar type of adherence noted between isolated gonococcal pili and latex beads (Buchanan, 1976) may both involve non-specific mechanisms. At least in the case of common type 1 pili, the effect of mannose or α -methyl mannoside on the adhesion of isolated filaments to 'polystyrene latex' beads should be assessed before any conclusions can be reached about the adhesion mechanisms involved.

The authors wish to thank Anne Strachan for typing the manuscript, Alex Ellis for technical assistance and Ian McKie for photographic services.

REFERENCES

- BRINTON, JR, C. C. (1959). Non-flagellar appendages of bacteria. *Nature, London* **183**, 782-786.
- BRINTON, JR, C. C. (1965). The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram-negative bacteria. *Transactions of the New York Academy of Sciences* **27**, 1003-1054.
- BUCHANAN, T. M. (1976). Attachment of purified gonococcal pili to latex spheres. In *Microbiology - 1976*, pp. 491-493. Edited by D. Schlessinger. Washington: American Society for Microbiology.
- DUGUID, J. P. & GILLIES, R. R. (1957). Fimbriae and adhesive properties in dysentery bacilli. *Journal of Pathology and Bacteriology* **74**, 397-411.
- DUGUID, J. P. & GILLIES, R. R. (1958). Fimbriae and haemagglutinating activity in *Klebsiella*, *Proteus* and *Chromobacterium*. *Journal of Pathology and Bacteriology* **75**, 519-560.
- DUGUID, J. P., SMITH, I. W., DEMPSTER, G. & EDMUNDS, P. N. (1955). Non-flagellar filamentous appendages ('fimbriae') and haemagglutinating activity in *Bacterium coli*. *Journal of Pathology and Bacteriology* **70**, 335-354.
- DUGUID, J. P., ANDERSON, E. S. & CAMPBELL, I. (1966). Fimbriae and adhesive properties in *Salmonella*. *Journal of Pathology and Bacteriology* **92**, 107-138.
- EVANS, D. G., SILVER, R. P., EVANS, JR, D. J., CHASE, D. G. & GORBACH, S. L. (1975). Plasmid-controlled colonisation factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. *Infection and Immunity* **12**, 656-667.
- EVANS, D. G., EVANS, D. J. & TJOA, W. (1977). Haemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhoea: correlation with colonisation factor. *Infection and Immunity* **18**, 330-337.
- FREER, J. H., ARBUTHNOTT, J. P. & BERNHEIMER, A. W. (1968). Interaction of staphylococcal α -toxin with artificial and natural membranes. *Journal of Bacteriology* **95**, 1153-1168.
- GOW, J. & WARDLAW, A. C. (1975). Iodination of a mixture of soluble proteins by the lactoperoxidase technique. *Biochemical and Biophysical Research Communications* **67**, 43-49.
- HASHIMOTO, M., TSUCHIMOTO, S., KATO, A., TAKEMOTO, T., YOSHINO, T., IKUTA, M. & NAKAJIMA, M. (1963*a*). Studies on structure and function of bacterial fimbriae. Report I. Demonstration of fimbriae in gram-negative enteric bacteria. *Bulletin of the Tokyo Medical and Dental University* **10**, 181-203.
- HASHIMOTO, M., TANIGUCHI, H., TAKEMOTO, T., YOSHINO, T., NAKAJIMA, M., HAGIWARA, I., TOBA, M. & NISHIDO, T. (1963*b*). Studies on structure and function of bacterial fimbriae. Report II. Adhesive properties of fimbriae. *Bulletin of the Tokyo Medical and Dental University* **10**, 493-506.
- HUBBARD, A. L. & COHN, Z. A. (1972). The enzymatic iodination of the red cell membrane. *Journal of Cell Biology* **55**, 390-405.
- JONES, G. W. (1977). The attachment of bacteria to the surfaces of animal cells. In *Receptors and Recognition*, Series B, vol. 3, *Microbial Interactions*, pp. 139-176. Edited by J. L. Reissig. London: Chapman & Hall.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- NOVOTNY, C., CARNAHAN, J. & BRINTON, JR, C. C. (1969). Mechanical removal of F pili, type I pili and flagella from Hfr and RTF donor cells and the kinetics of their reappearance. *Journal of Bacteriology* **98**, 1294-1306.
- OLD, D. C. (1972). Inhibition of the interaction between fimbrial haemagglutinins and erythrocytes by D-mannose and other carbohydrates. *Journal of General Microbiology* **71**, 149-157.
- OLD, D. C. & PAYNE, S. B. (1971). Antigens of the type-2 fimbriae of salmonellae: 'cross-reacting material' (CRM) of type-1 fimbriae. *Journal of Medical Microbiology* **4**, 215-225.
- SALIT, I. E. & GOTSCHLICH, E. C. (1977). Type 1 *Escherichia coli* pili: characterisation of binding

- to monkey kidney cells. *Journal of Experimental Medicine* **146**, 1182–1194.
- STECK, T. L. (1974). Preparation of impermeable inside-out and right-side out vesicles from erythrocyte membranes. In *Methods in Membrane Biology*, vol. 2, pp. 245–278. Edited by E. D. Korn. New York: Plenum Press.
- SWANSON, J. (1973). Studies on gonococcus infection. IV. Pili; their role in the attachment of gonococci to tissue culture cells. *Journal of Experimental Medicine* **137**, 571–589.